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Isolation, preconcentration and determination of rhamnolipids in aqueous samples by dispersive liquid–liquid microextraction and liquid chromatography with tandem mass spectrometry

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ABSTRACT

An analytical method based on liquid chromatography–tandem mass spectrometry (LC–MS–MS) was developed for the determination of rhamnolipids. A dispersive liquid–liquid microextraction (DLLME) procedure was used to isolate and concentrate target compounds from aqueous samples collected from surface water, sewage treatment plant effluent and cultivation of microbial culture. Development of the DLLME procedure included optimization of several important parameters such as kind and volume of extracting and dispersing solvents as well as sample pH. Under optimized conditions a two-step extraction with sonication was used. Chloroform was applied as the extracting and acetone as the dispersing solvent. The recoveries of the analytes were 70–87%. Matrix effects investigated for the analytes revealed existence of ionization enhancement for both mono- and dirhamnolipids.

1. Introduction

Surfactants are important compounds which are used in a wide spectrum of industrial, agricultural and domestic applications. Some surfactants are produced naturally and therefore are named biosurfactants. Biosurfactants are amphipatic molecules produced by a wide variety of bacteria, yeast and filamentous fungi from various substrates including sugars, glycerol, oils, hydrocarbons and agricultural wastes. In contrast to surfactants based on petroleum feedstock, biosurfactants are produced in mild conditions and are better biodegradable. Moreover, their toxicity is lower and they do not permanently pollute the environment. Because of their unique properties interest in their industrial production increased considerably [1,2].

Currently, among the most promising biosurfactants rhamnolipids found large interest. Rhamnolipid molecules consist of one or two rhamnose rings connected to one or two β-hydroxy fatty acids. The fatty chains consist of 8–14 carbon atoms with or without a double bond [3,4]. Usually a mixture of different rhamnolipids is produced. Composition of this mixture depends mainly on bacterial species. *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Pseudomonas oleovorans*, *Pseudomonas chlororaphis* and other species have been reported in the literature [3,5–7]. However, the most promising bacteria strains belong to *P. aeruginosa*. *P. aeruginosa* produce mainly two rhamnolipids: monorhamnolipid with two fatty acid containing 10 carbon atoms each (RhaC10C10) and dirhamnolipid with the same composition of fatty acid chain (RhaRhaC10C10). Ratio of RhaC10C10 to RhaRhaC10C10 and composition of other rhamnolipids present in the mixture produced by bacteria depends on bacteria strain and carbon source used in the experiment [1,7,8].

Rhamnolipids exhibit promising applications in industry, agriculture and other fields [9,10]. They are used in environmental clean up (removing oil spills, bioremediation), agricultural formulas (wetting agents, sticker and dispersal agents for fungicides, pesticides and nutrient sprays), pharmaceuticals (healing in skin diseases), food products (enhancing the volume of bakery products, stabilizing dough and batter), toiletries and household cleaners (shampoos, soaps and detergents) [1,11–13].

The aim of this work is to demonstrate the possibility of use of dispersive liquid–liquid microextraction (DLLME) in analysis of rhamnolipids. DLLME is a newly developed extraction technique which has been applied mainly for isolation of environmental contaminants from the water matrix [14,15]. It has been used for analysis of herbicides [16–18], alkylphenols [19,20] or polycyclic aromatic hydrocarbons [14,21]. Not many application were devoted to isolation of compounds from non-environmental samples. It has been used for isolation of pesticide from fruits and

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Table 1
Formulae of the analytes and MS parameters used for their detection.

Structure of analyte	Precursor ion $[M-H]^-$ m/z	Declustering potential [V]	MRM transitions (precursor ion $m/z \rightarrow$ product ion m/z)			
			MRM 1 (analytical)	Collision energy [V]	MRM 2 (confirmatory)	Collision energy [V]
Rha-C10-C10	503	80	503 → 169	28	503 → 333	21
Rha-Rha-C10-C10	649	85	649 → 169	35	649 → 479	29

vegetable [22,23], antibiotics from honey [24] or cholesterol from food samples [25].

2. Materials and methods

2.1. Reagents and chemicals

Mixture of rhamnolipids (JBR 425) was purchased from Jeneil Biosurfactant Company (Saukville, WI, USA) as 25% solution in water. MS-grade acetonitrile was from J.T. Baker (Deventer, The Netherlands). Ammonium acetate used as mobile phase additive was purchased from Sigma–Aldrich (St. Louis, MO, USA). Water was prepared by reverse osmosis in a Demiwa system from Watek (Ledec nad Sazavou, Czech Republic), followed by double distillation from a quartz apparatus. Only freshly distilled water was used.

All of the reagents used as the extracting solvents in the experiments were of analytical grade. Chloroform and trichloroethane were from Sigma–Aldrich. Trichloroethylene and tetrachloroethylene were from Merck (Darmstadt, Germany). Analytical grade acetone and MS-grade methanol used as dispersing solvents were obtained from J.T. Baker.

The reagents used for adjustment of sample solutions pH in testing of sample pH effect were of analytical grade. Hydrochloric acid applied for pH adjustment was from POCh (Gliwice, Poland). The buffer solutions were prepared from formic acid, acetic acid and monosodium phosphate (all from POCh) adjusted to required pH with sodium hydroxide from POCh. All reagents used for preparation of the test medium were also purchased from POCh.

2.2. Liquid chromatography–mass spectrometry

The chromatographic system UltiMate 3000 RSLC from Dionex (Sunnyvale, CA, USA) was used. Five μL samples were injected into a 50 mm × 2.0 mm I.D. analytical column packed with 3 μm Luna C18 from Phenomenex (Torrance, CA, USA). The mobile phase used for the analysis consisted of 5 mmol L^{-1} ammonium acetate in water (A) and acetonitrile (B) at a flow rate of 0.3 mL min^{-1}. The following gradient was used: 0 min 55% B; 4 min 55% B; 4.5 min 90% B; 7 min 90% B. The LC column effluent was directed to the electrospray ionisation source (Turbo Ion Spray).

The HPLC system was connected to the API 4000 QTRAP triple quadrupole mass spectrometer from AB Sciex (Foster City, CA, USA). The Turbo Ion Spray source operated in negative ion mode. The dwell time for each mass transition detected in the MS/MS multiple reaction monitoring mode was set to 20 ms. All rhamnolipids were detected using the following settings for the ion source and mass spectrometer: curtain gas 10 psi, nebulizer gas 40 psi, auxiliary gas 45 psi, temperature 450 °C, ion spray voltage – 3500 V and collision gas set to medium. The mass spectrometer parameters specific for the analytes are summarised in Table 1 and mass spectra are presented in Fig. 1.

2.3. Dispersive liquid–liquid microextraction procedure

Dispersive liquid–liquid microextraction was used for isolation of the analytes. Six millilitres of water sample were placed in a 10 mL glass test tube with a conical bottom. The pH of the sample was adjusted to 2 with HCl. One mL of acetone (dispersing solvent) containing 70 μL of chloroform (extracting solvent) was injected rapidly into the sample solution using a 2 mL syringe. In this step, the extraction solvent was dispersed into the aqueous sample as very fine droplets and a cloudy solution was formed in the test tube. Then, the mixture was sonicated for 1 min and centrifuged for 10 min at 4300 rpm. The dispersed fine particles of extraction phase were sedimented in the bottom of the test tube. The sedimented phase was withdrawn with a 100 μL micro-syringe. The sample was extracted once again. The combined sedimented extracts were evaporated to dryness with a gentle nitrogen purge and reconstituted to 40 μL of a mixture of acetonitrile: water (3:1; v/v) and injected into HPLC for analysis.

2.4. Sample collection and handling

The water samples were collected to clean glass bottles prerinsed with the sample. About one litre of the sample was taken from both the centre of the river current and sewage treatment plant effluent. The samples were collected and analysed in the same day.

2.5. Cultivation and rhamnolipid production

The microbial strain *P. aeruginosa Pa 10TK* was used in the experiments. The strain was isolated from soil contaminated by a crude oil. The contaminated samples were collected from petrol station. Culture medium used throughout the study consisting per 1 g of carbon source was: $NaNO_3$ 137.5 mg; $MgSO_4 \cdot 7H_2O$ 22 mg; KCl 55 mg; NaCl 55 mg; $CaCl_2 \cdot 2H_2O$ 2.75 μg; $FeSO_4 \cdot 7H_2O$ 27.5 μg; $ZnSO_4 \cdot 7H_2O$ 82.5 μg; $MnSO_4 \cdot H_2O$ 82.5 μg; H_3BO_3 16.5 μg; $CoCl_2 \cdot 6H_2O$ 8.3 μg; $CuSO_4 \cdot 5H_2O$ 8.3 μg; $NaMoO_4 \cdot 2H_2O$ 5.5 μg and H_3PO_4 (d = 1.71 [g cm^{-3}]) 110 μL [26]. Medium assures the most profitable relation C:Fe, C:N and C:P. The pH of the medium was 7.2. A liquid culture was started by adding a loop full of cells from an agar plate to a 250 mL Erlenmeyer flask containing 50 mL of medium. After approximately 24 h a few mL (in the range 3–5 mL) of this liquid culture was used for the inoculation of the final culture to reach optical density of ca. 0.1 (this corresponds to 1×10^8 cells per mL). The microbial growth was monitored through culture densities, measuring absorption spectrophotometrically at 600 nm (1601PC spectrophotometer from Shimadzu, Tokyo, Japan). Glucose was used as carbon source. Final culture contained 100 mL medium, 1 mL of glucose solution and a few ml of culture broth. Samples were incubated at 25 °C, shaken at 120 rpm for 7 days.

2.6. Method performance

Calibration curve range of the method was tested in a wide range for both RhaC10C10 and RhaRhaC10C10. Seven calibration levels were included in each calibration line. The calibration was performed for river water samples spiked after extraction with JBR 425 standard containing RhaC10C10 and RhaRhaC10C10 rhamnolipids.

The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated on the basis of signal to noise (S/N) ratio. The S/N = 3 was used for calculation of LOD and the S/N = 10 for calculation of

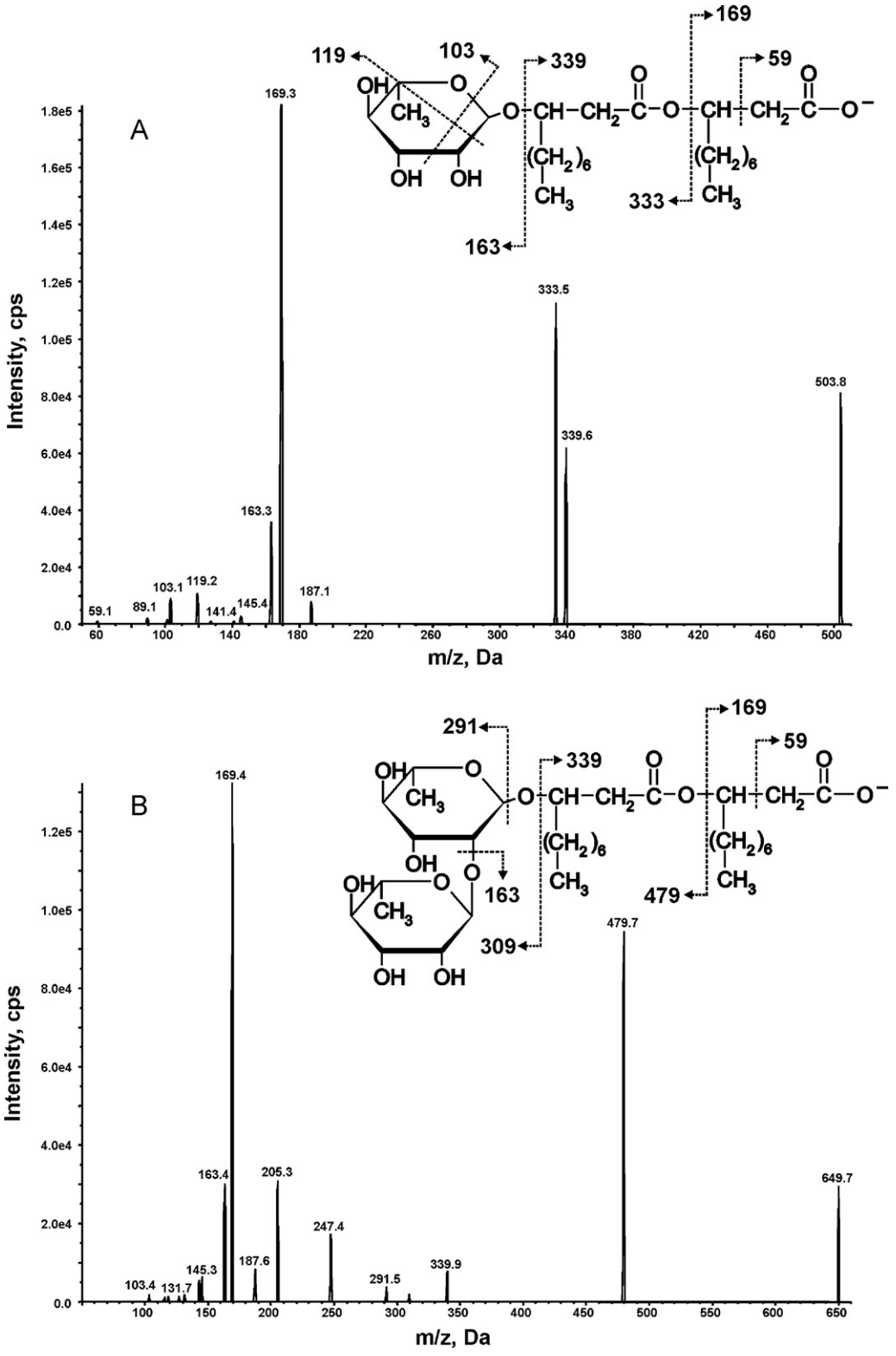

Fig. 1. Mass spectra of (A) monorhamnolipid RhaC10C10, (B) dirhamnolipid RhaRhaC10C10.

LOQ. The LOD and LOQ were determined for river water samples spiked after extraction.

The matrix effect was determined according to procedure proposed by Matuszewski et al. [27]. Three sets of data were prepared. Set A included the results gained for standards. Set B contained data gathered for river water samples spiked after extraction and set C river water samples spiked before extraction. The matrix effect (ME), recovery of the extraction procedure (RE) and overall process efficiency (PE) were calculated according to Matuszewski et al. [27] as follows:

$$\text{ME} \quad (\%) = \frac{B}{A} \times 100 \tag{1}$$

$$\text{RE}\ \ (\%) = \frac{C}{B} \times 100 \tag{2}$$

$$\text{PE}\ \ (\%) = \frac{C}{A} \times 100 = \frac{\text{ME} \times \text{RE}}{100} \tag{3}$$

The *A*, *B* and *C* in Eq. (1), (2) and (3) are peak areas obtained for sets A, B and C, respectively. The study was performed at two levels of concentration and two sets of peak areas (A, B and C) were obtained. The upper spiking level before extraction was 3 μg L^{-1} and the lower spiking level was 0.3 μg L^{-1}. Precision of the method was calculated from the results gained for samples spiked before extraction.

3. Results and discussion

3.1. Selection of the extracting solvent and the dispersing solvent

Selection of the extracting solvent and the dispersing solvent is the first step in optimization of any DLLME procedure. Usually a few pairs of extracting and dispersing solvents are tested to enable their proper selection. Two different types of optimization can be found in the literature. In the first approach a few extracting solvents are tested with one dispersing solvent and on the basis of this experiment the extracting solvent is selected. Then the selected extracting solvent is tested with another dispersing solvents. The pair of the extracting solvent and the dispersing solvent is chosen on the basis of two consecutive experiments [14–17]. In the second approach all combinations of the extracting solvent and the dispersing solvent are tested in one experiment [18,19]. This type of solvent selection procedure is time-consuming. However, one cannot exclude any solvent pair (including potentially the best choice) which is possible in the first approach. Thus, the second procedure was used in this study.

Three dispersing solvents and four extracting solvents were tested for extraction of rhamnolipids from the water matrix. The dispersing solvents used in DLLME procedure optimization were acetone, acetonitrile and methanol. The four extracting solvents selected for optimization were: chloroform, trichloroethane, trichloroethylene and tetrachloroethylene. The results obtained during selection of the extracting and dispersing solvent pair are presented in Fig. 2. The results obtained in this experiment showed clearly that extraction efficiency is much higher for monorhamnolipid RhaC10C10 than for dirhamnolipid RhaRhaC10C10. The reason for this is probably more polar nature of RhaRhaC10C10 than RhaC10C10. Additional sugar ring in dirhamnolipids should enhance their solubility in water in comparison to monorhamnolipids. Better solubility in water results in difficult extraction of RhaRhaC10C10 from the water matrix. As a result it was decided to chose the best extraction solvent pair on the basis of RhaRhaC10C10 recovery. This was achieved for chloroform with acetone. Moreover, this solvent system enabled also obtaining the highest recovery for monorhamnolipid RhaC10C10.

3.2. Effect of sample pH

Rhamnolipids belong to surfactants containing carboxylic group in their molecules. Therefore they can be present in water in both ionized and unionized form. The ionized form is easily soluble in water and cannot be extracted to chloroform. The unionized form can be extracted. Thus, it should be advantageous to change equilibrium in water by pH adjustment.

Recovery of rhamnolipids was tested in pH range from 1 to 7 as it was expected that their ionization would be reduced at low pH. Hydrochloric acid was utilized to adjust sample pH to 1 or 2. Formic buffer was applied at pH 3, acetic buffer was used at pH 4 and 5 and phosphoric buffer was employed at pH 7.

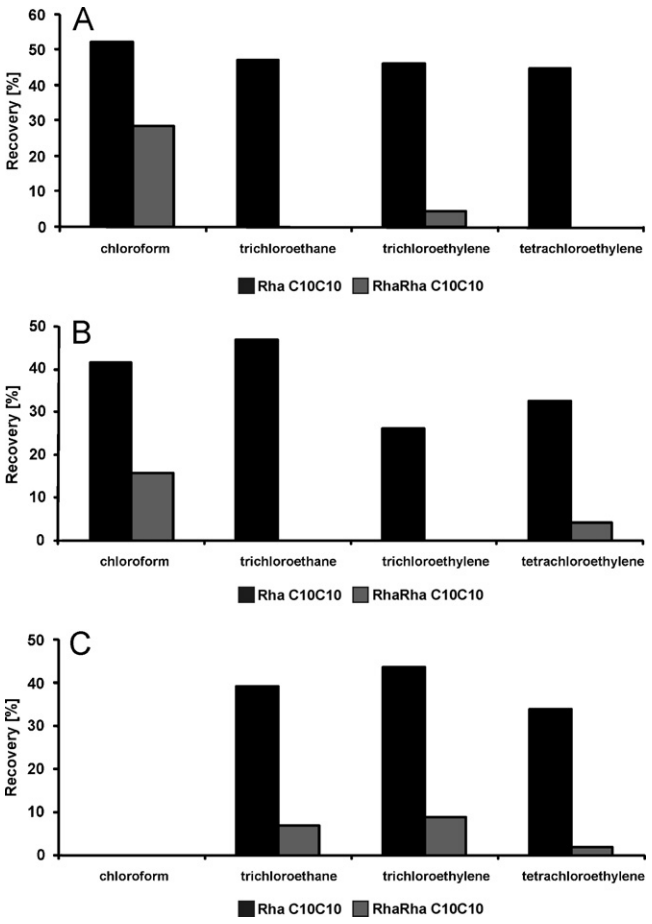

Fig. 2. Recoveries of RhaC10C10 and RhaRhaC10C10 for different pairs of the dispersing and extracting solvents. The dispersing solvents were: (A) acetone, (B) acetonitrile, (C) methanol. Extraction conditions: 6 mL of water sample, 1 mL of dispersing solvent containing 50 μL of extracting solvent. No results were presented for chloroform with methanol as no phase separation took place for this pair of solvents.

The results collected in this experiment (Fig. 3) confirmed the above-mentioned statement. Indeed, higher recovery of the analytes was obtained for low pH of the sample. Thus, acidification step was introduced in further experiments. All the following samples were acidified to pH 2.

3.3. Effect of volumes of the extracting solvent and the dispersing solvent

Optimization of volumes of the extracting solvent and the dispersing solvent was a further step in development of the DLLME

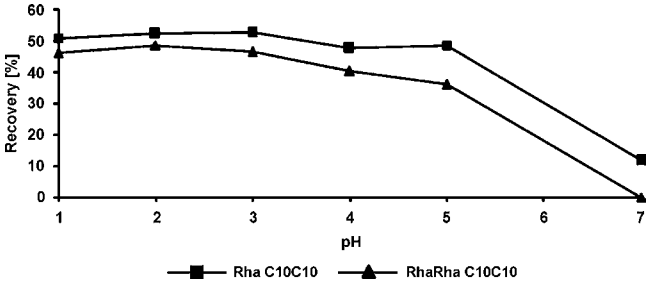

Fig. 3. Effect of sample pH on recoveries of RhaC10C10 and RhaRhaC10C10. Extraction conditions: 6 mL of water sample adjusted to selected pH with 1 mL of hydrochloric acid or a buffer solution, 1 mL of dispersing solvent (acetone) containing 50 μL of extracting solvent (chloroform).

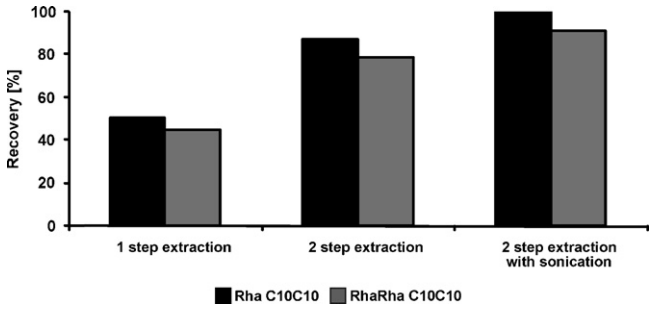

Fig. 4. Effect of sonication and two-step (double) extraction on the recovery of RhaC10C10 and RhaRhaC10C10. Extraction conditions: 6 mL of water sample adjusted to pH 2 with 100 μL of hydrochloric acid, 1 mL of dispersing solvent (acetone) containing 70 μL of extracting solvent (chloroform). Second step of extraction was performed in the same way like the first one.

procedure. Both these volumes can influence formation of dispersion and thus should be optimized.

Effect of the dispersing solvent volume was studied for four different acetone volumes: 0.5, 1.0, 1.5 and 2.0 mL. The extracting solvent volume was kept constant at 50 μL. The results collected in this experiment show considerable dependence of the dispersing solvent volume on extraction efficiency. The lowest recoveries were obtained for 0.5 mL and 2.0 mL. This can be ascribed to two different effects. Low volume of the dispersing solvent (0.5 mL) results in problems with formation of stable dispersion. High volume of the dispersing solvent (2.0 mL) leads to better solubility of chloroform in the water:acetone mixture. This effect resulted in lower volume of collected sedimented phase and also in lower recovery of the analytes.

The highest recovery of both RhaC10C10 and RhaRhaC10C10 was obtained for 1.0 mL and 1.5 mL of the dispersing solvent. Higher recovery was noted for RhaC10C10 when 1.5 mL of acetone was used. Nevertheless, 1.0 mL of acetone resulted in better recovery for RhaRhaC10C10. One mL of acetone was selected for further experiments as this volume assured better extraction of RhaRhaC10C10 – the analyte for which recovery in DLLME was more problematic.

Effect of the extracting solvent volume was tested for six volumes of chloroform. The range from 40 to 90 μL of the extracting solvent volume was tested. The results obtained in this experiment proved influence of the extracting solvent volume on the recovery of the analytes. The dependence curve was non-linear with maximum at about 70 μL. This volume was applied in further experiments.

3.4. Effect of sonication and two-step extraction

There were no satisfactory results obtained in the former experiment as the recovery of the analytes was about 50%. Thus, two additional steps in extraction procedure were tested: sonication and two-step extraction. Sonication should improve formation of dispersion. Two-step extraction should improve recovery of the analytes in analogy to liquid–liquid extraction. Both these additional factors were tested in one experiment. The results presented in Fig. 4 confirm positive effect of both sonication and two-step extraction. The obtained recovery was high and the procedure with sonication and two-step extraction was used for testing real water samples.

3.5. Real samples analysis

Before real water samples could be analysed, several additional tests were required. Calibration curve range of the method was calculated for evaporated sample extracts spiked with standard solutions at seven different concentration levels. Correlation coefficients obtained here were at least 0.997 (Table 2). Also limit of detection and limit of quantitation calculated on the basis of signal to noise ratio were satisfactory (Table 2).

Table 2
Calibration curve range and correlation coefficient, limit of quantitation, limit of detection, matrix effect, recovery, process efficiency, precision and enrichment factor obtained during method performance testing on spiked river water.

Parameters	RhaC10C10	RhaRhaC10C10
Calibration curve range [μg L^{-1}]	11–900	14–1100
Correlation coefficient (r^2)	0.9998	0.9968
Limit of quantitation [ng L^{-1}]	0.010	0.012
Limit of detection [ng L^{-1}]	0.003	0.004
Matrix effect (ME) at 0.3 μg L^{-1} [%]	126	113
Recovery (RE) at 0.3 μg L^{-1} [%] (n = 5)	87	72
Process efficiency (PE) at 0.3 μg L^{-1} [%] (n = 5)	108	78
Precision (RSD) at 0.3 μg L^{-1} [%] (n = 5)	4	3
Matrix effect (ME) at 3 μg L^{-1} [%]	122	113
Recovery (RE) at 3 μg L^{-1} [%] (n = 5)	80	70
Process efficiency (PE) at 3 μg L^{-1} [%] (n = 5)	98	79
Precision (RSD) at 3 μg L^{-1} [%] (n = 5)	3	9
Enrichment factor	154	118

The matrix effect, recovery, process efficiency and precision of the method were also tested. The results from this experiment (Table 2) show influence of matrix effect. The matrix effect was from 113% for RhaRhaC10C10 to 126% for RhaC10C10 which means that signal enhancement was observed for both the analytes. Recoveries of the tested compounds were lower than those calculated for HPLC-grade water. However, this was partially compensated by signal enhancement. Thus, overall process efficiency from 78% for RhaRhaC10C10 to 108% for RhaC10C10 was satisfactory. The precision of the method was also acceptable for both the analytes. This allowed for the use of the optimized method in analysis of three water samples of different origin.

Presence of rhamnolipids in the environment enables biodegradation of many pollutants including oils. They are also commercially used for bioremediation. Rhamnolipids are produced by many bacterial strains to lower surface tension and enable use of oils as carbon source. Their concentration in the environment was not tested as they are non-toxic. The two rhamnolipids tested in this study were found in the water sample taken from the Warta River (Table 3). Their concentration is relatively low in comparison to the most popular commercially used surfactants like linear alkylbenzene sulfonates [28], alcohol ethoxylates [29] and alkylphenol ethoxylates [29]. This is in accordance with their relatively lower production and usage. However, commercial usage of rhamnolipids is not their sole source in the river water. Rhamnolipids are produced by many bacterial strains and can be directed to the environment from sewage treatment plants as a by-product of the biodegradation process. Indeed, the water effluent taken from the sewage treatment plant and tested for rhamnolipids contained high level of these surfactants (Table 3). The proportion of the two tested rhamnolipids, however, differed considerably from that noted for river water sample. This can be a result of different bacterial strains producing rhamnolipids in the sewage treatment plant. Typically, similar amounts of RhaC10C10 and RhaRhaC10C10 are produced by *P. aeruginosa* strains. This was also confirmed in the test carried

Table 3
Content of rhamnolipids RhaC10C10 and RhaRhaC10C10 in water samples.

Sample	Concentration [μg L^{-1}]	
	RhaC10C10	RhaRhaC10C10
River water	0.280 ± 0.027	0.200 ± 0.010
Sewage treatment plant effluent	0.803 ± 0.081	0.104 ± 0.007
Cultivation of *Pseudomonas aeruginosa Pa 10TK* strain	0.101 ± 0.009	0.131 ± 0.012

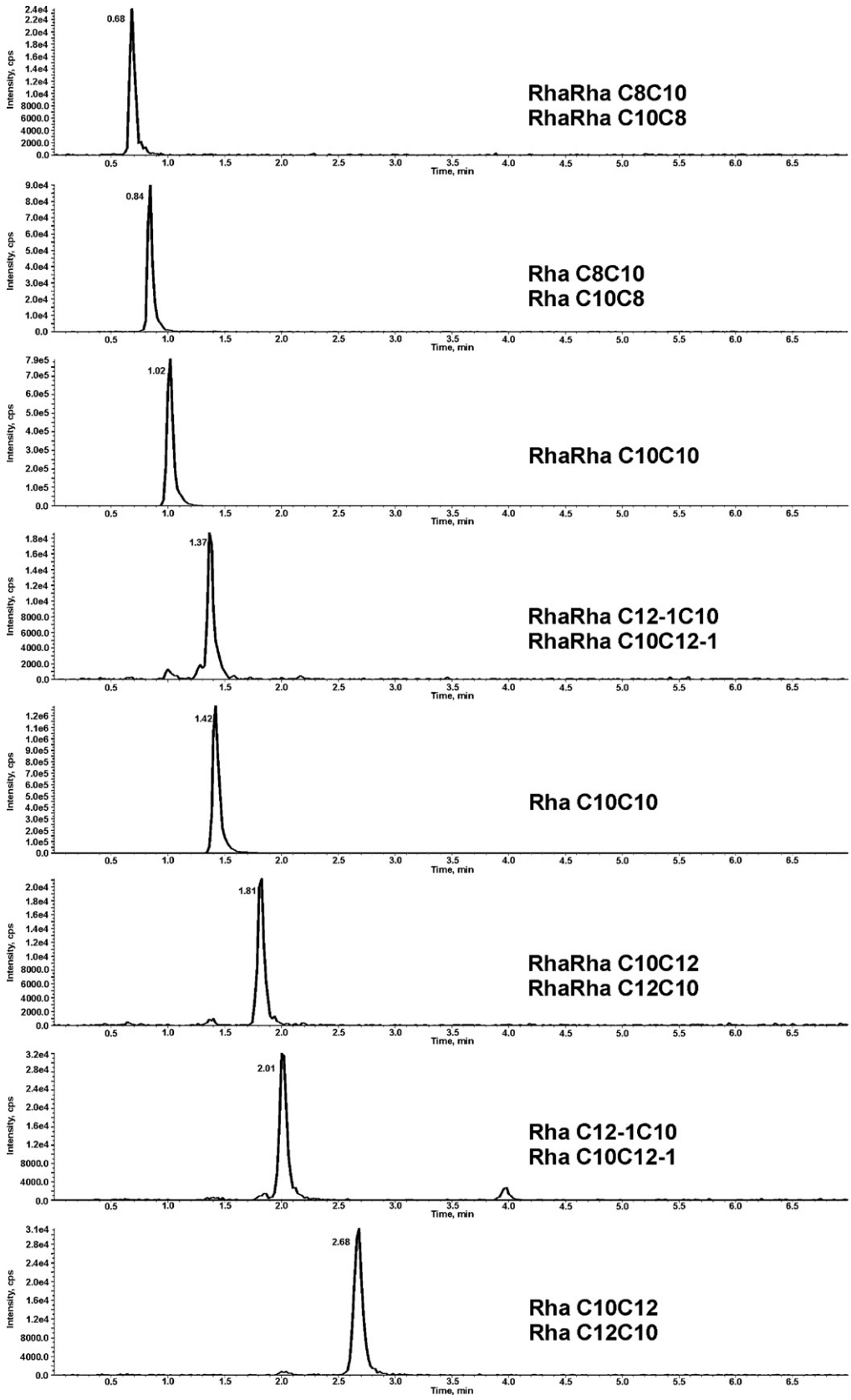

Fig. 5. Chromatograms of RhaC10C10, RhaRhaC10C10 and other mono- and dirhamnolipids produced by *Pseudomonas aeruginosa Pa 10TK*.

out in our lab (Table 3) where similar amount of RhaC10C10 and RhaRhaC10C10 were produced by *P. aeruginosa Pa 10TK* strain.

The procedure used in this experiment allowed for testing presence of rhamnolipids in different samples. It can be also used for analysis of the profile of rhamnolipids produced by different bacterial strains. Several other rhamnolipids were also found in the mixture produced by *P. aeruginosa Pa 10TK* strain (Fig. 5). This shows the potential of the newly developed method for extraction of different rhamnolipids. Also, the scaled-up version of the extraction procedure could possibly be used for isolation of rhamnolipids in commercial production of these surfactants.

4. Conclusion

This paper outlined the successful development and application of the DLLME for analysis of rhamnolipids in water samples. The application of DLLME to this group of analytes has not been reported before. The developed method offers several advantages such as simplicity, low cost, high enrichment and short time of sample preparation. Good performance of this method in analysis of real water samples demonstrates the possibility of its use in routine analysis.

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